



Immunoenhancing effects of Montanide™ ISA oil-based adjuvants on recombinant coccidia antigen vaccination against *Eimeria acervulina* infection[☆]

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ABSTRACT

The current study was conducted to investigate the immunoenhancing effects of Montanide™ adjuvants on protein subunit vaccination against avian coccidiosis. Broiler chickens were immunized subcutaneously with a purified *Eimeria acervulina* recombinant profilin protein, either alone or mixed with one of four adjuvants (ISA 70 VG, ISA 71 VG, ISA 201 VG or ISA 206 VG), and body weight gains, fecal oocyst shedding, and humoral and innate immune responses were evaluated following oral challenge infection with live *E. acervulina* oocysts. Immunization with profilin plus ISA 70 VG or ISA 71 VG increased body weight gains compared with vaccination with profilin alone. Profilin plus ISA 71 VG also reduced fecal oocyst shedding compared with vaccination in the absence of adjuvant. All adjuvants enhanced profilin serum antibody titers. Increased levels of gene transcripts encoding IL-2, IL-10, IL-17A, and IFN- γ , but decreased levels of IL-15 mRNAs, were seen in intestinal intraepithelial lymphocytes of chickens immunized with profilin plus adjuvants compared with immunization with profilin alone. Finally, increased infiltration of lymphocytes, especially CD8⁺ lymphocytes at the site of immunization was observed in birds given profilin plus ISA 71 VG compared with profilin alone. These results demonstrate that vaccination with the *E. acervulina* profilin subunit vaccine in combination with Montanide™ adjuvants enhances protective immunity against avian coccidiosis.

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1. Introduction

Coccidiosis is a major poultry disease of great economic importance that is caused by at least seven species of *Eimeria* apicomplexan protozoa that infect the intestinal mucosa (Lillehoj et al., 2004). Afflicted animals exhibit a variety of clinical manifestations including nutrient malabsorption, inefficient feed utilization, impaired body weight gain and, in severe cases, mortality. Although

prophylactic medication is the predominant method used to suppress flock infections, new disease control strategies are needed due to the emergence of drug-resistant field strains of *Eimeria* and increasing consumer demands for drug-free poultry meat.

Because chickens infected with *Eimeria* spp. develop protective immunity against reinfection by the homologous parasite, immunization with parasite vaccines represents a viable method to control coccidiosis (Lillehoj et al., 2000a). Live coccidia vaccines are commercially available, but cross protection against heterologous *Eimeria* spp. or antigenic variants that are not present in the vaccine formulation is poor. Studies using recombinant protein vaccines derived antigens common to multiple coccidia species to stimulate broad-spectrum immunity have shown limited success, mainly because of their low antigenicity, inadequate stimulation of protective host immunity, and/or restricted expression during the parasite life-cycle (Lillehoj et al., 2000b, 2005a; Ding et al., 2004). Therefore, interest has been generated in using immunostimulators, such as vaccine adjuvants, to enhance the immunogenicity of recombinant coccidia subunit vaccines (Lillehoj et al., 2005a).

Since the first description of adjuvants as immune enhancers in 1925 (Ramon, 1925), many different types of chemical compounds and formulations have been demonstrated to be effective in augmenting humoral and cell-mediated immune responses (Newman and Powell, 1995; Bowersock and Martin, 1999). Among the most frequently used adjuvants for human and veterinary vaccines are aluminum salts (alum) and oil-based emulsions (Freund et al., 1948; Gupta et al., 1995; Bowersock and Martin, 1999). Alum has been incorporated in several human vaccines and is the only adjuvant approved for such use in the United States. Although the exact mechanism of action of alum is unknown, physical binding to antigens, retention of antigens at injection sites, and antigen delivery to lymph nodes are known to play a contributing role. In animal models, novel adjuvants which are more effective than alum in enhancing antibody and/or cell-mediated immune responses have been described (Lawrence et al., 1997; Aucouturier and Ganne, 2000). In particular, the Montanide™ ISA series of water-in-oil emulsion adjuvants have shown superior efficacy with a variety of human and animal vaccines (Cox et al., 2003). However, even if Montanide™ adjuvants have not previously been tested for their ability to enhance the immunogenicity of avian coccidiosis subunit vaccines, Montanide™ ISA 70 VG has already been demonstrated as safe and efficient in numerous poultry disease models (Aucouturier and Ganne, 2000; Belloc et al., 2008).

Therefore, this study was conducted to evaluate the effectiveness of four Montanide™ adjuvants in promoting protective immunity against avian coccidiosis following vaccination with profilin, an actin-regulatory protein that is expressed by multiple *Eimeria* spp. at all asexual stages including sporozoites, merozoites, and sporulated oocysts (Song et al., 2000).

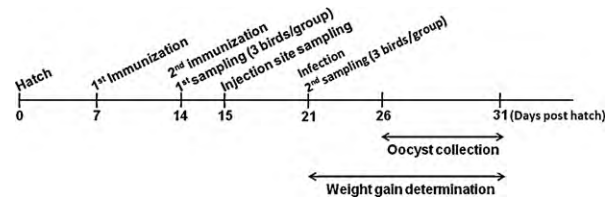


Fig. 1. Schematic outline of the experimental design.

2. Materials and methods

2.1. Chickens

One-day-old broiler chickens (Ross/Ross) were obtained from Longenecker's Hatchery (Elizabethtown, PA), housed in the Petersime starter brooder units, and provided with feed and water *ad libitum*. All experiments were approved by the Beltsville Agricultural Research Center Small Animal Care and Use Committee.

2.2. Recombinant profilin protein

The profilin gene was originally cloned by immunoscreening an *Eimeria acervulina* cDNA library using a rabbit antiserum against *E. acervulina* merozoites (Song et al., 2000). The 1086-base pair 3-1E (profilin) cDNA was subcloned into the pMAL plasmid with an NH₂-terminal maltose-binding protein epitope tag and a Factor Xa protease cleavage site between maltose-binding protein and profilin (Ding et al., 2004). Transformed *Escherichia coli* DH5α bacteria were grown overnight to mid-log phase, induced with 1.0 mM of isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C, collected by centrifugation, and disrupted by sonication on ice (Misonix, Farmingdale, NY). The recombinant profilin protein was isolated on an amylose affinity column (New England Biolabs, Beverly, MA) according to the manufacturer's instructions, digested with Factor Xa to release profilin from the solid phase, and repassed through the amylose column to remove any contaminating maltose-binding protein. Final purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with profilin-specific rabbit antibody (Ding et al., 2004).

2.3. Adjuvants

ISA 70 VG and ISA 71 VG are ready to use adjuvant rendering water-in-oil (W/O) emulsions, ISA 201 VG and ISA 206 VG are ready to use adjuvant rendering water-in-oil-water (W/O/W) double emulsions. Purified profilin was mixed with ISA 70 VG and ISA 71 VG at a 30:70 ratio (w:w, profilin:adjuvant), with ISA 206 VG and ISA 201 VG at 50:50 ratio, as recommended by the adjuvant manufacturer.

2.4. Parasites

The strain of *E. acervulina* used in this study was originally developed and maintained at the Animal Par-

asitic Diseases Laboratory of the Animal and Natural Resources Institute (Beltsville, MD) (Song et al., 2000). Sporulated oocysts were cleaned by flotation on 2.5% sodium hypochlorite, washed three times with PBS, and enumerated using a hemocytometer before giving to birds.

2.5. Experimental infections and evaluation of protective immunity

Chickens were randomly divided into 7 groups of 20 birds each (Fig. 1). At 1 week of age, the chickens were immunized subcutaneously with 50 µg of profilin alone or profilin–adjuvant mixture. Control chickens were injected with PBS alone. At 7 d post-primary immunization, secondary immunizations were given subcutaneously with 50 µg of profilin–adjuvant mixture. At 7 d post-secondary immunization, chickens were challenged orally with 1.0×10^4 sporulated *E. acervulina* oocysts. Body weights were measured between 0 and 10 d post-secondary infection. Fecal oocysts numbers were counted between 5 and 10 d post-secondary infection using a McMaster chamber as described (Ding et al., 2004).

2.6. Profilin serum antibody responses

At 7 d post-primary and 7 d post-secondary immunization, blood was collected by cardiac puncture from 3 birds/group under euthanasia, sera were obtained by low speed centrifugation, and used in an ELISA to measure profilin-specific antibody responses as described (Ding et al., 2004). Microtiter plates were coated overnight with 10 µg/well of purified recombinant profilin, washed with PBS–0.05% Tween, and blocked with PBS–1% BSA. Serum samples were incubated with continuous shaking, the plates were washed, and bound antibody was detected with peroxidase-conjugated rabbit anti-chicken IgG and peroxidase-specific substrate (Sigma, St. Louis, MO). OD values at 450 nm were measured with an automated microplate reader (Bio-Rad, Richmond, CA).

2.7. Cytokine mRNA levels in intestinal intraepithelial lymphocytes (IELs)

At 7 d post-secondary immunization, a segment of the intestinal duodenum was excised, cut open longitudinally, gently washed with ice-cold Hank's Balanced Salt Solution (Sigma), and IELs were isolated by density gradient centrifugation as described (Hong et al., 2006). IEL total RNA was isolated, 5.0 µg were treated with 1.0 U of DNase I and 1.0 µl of 10× reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 µl of stop solution was added to inactivate DNase I, and the mixture was heated at 70 °C for 10 min. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. PCR amplification and detection were carried out using equivalent amounts of total RNA and oligonucleotide primers for IL-2, IL-10, IL-15, IL-17A, TGF-β4, and IFN-γ (Table 1) with the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves were generated using log₁₀ diluted standard RNAs and the lev-

els of individual transcripts were normalized to those of GAPDH by the Q-gene program (Muller et al., 2002; Hong et al., 2006). Each analysis was performed in triplicates. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) values for the amplification products were calculated by pooling values from all samples in that experiment.

2.8. Indirect immunofluorescence microscopy

Skin samples from 3 birds/group were obtained from the injection site at 1 d post-secondary immunization from chickens immunized with profilin alone or profilin-ISA 71 VG mixture. The samples were immediately embedded in optimum cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and stored at –20 °C. Immunofluorescence staining was conducted as described (Fritschy et al., 1992). Five micrometers sections were mounted on pre-cleaned slides, fixed in acetone for 20 min at 4 °C, and blocked with 10% normal horse serum for 20 min at room temperature. Mouse monoclonal antibodies K55 (whole lymphocytes), K1 (macrophages and thrombocytes), and T lymphocyte subpopulations (CD8⁺, TCRγδ⁺, TCRαβ⁺) (Lillehoj et al., 2005b) were added, incubated at room temperature for 2 h, the slides were washed with PBS, and incubated with Alexa Fluor 488-labeled chicken anti-mouse IgG secondary antibody (1:500 dilution; Invitrogen, Carlsbad, CA) for 2 h at room temperature. The slides were mounted with Fluoromount-G and observed by confocal laser scanning immunofluorescence microscopy (LSM 510 META, Carl Zeiss, Thornwood, NY).

2.9. Statistical analysis

All data were subjected to one-way analysis of variance using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Mean ± S.D. values were compared using the Tukey's test and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effects of vaccination with profilin plus Montanide™ adjuvants on body weight gain and fecal oocyst shedding

Our previous report documented that subcutaneous vaccination of chickens with 50 µg of *Eimeria* recombinant profilin protein provided partial protection against subsequent challenge infection with live parasites, with decreased fecal oocyst shedding but no increase in body weight gain compared with unimmunized controls (Ding et al., 2004). Higher profilin doses were effective both in reducing oocyst shedding and restoring body weight gain. Therefore, we chose this suboptimal vaccine dose to evaluate the effect of profilin vaccination in the presence of Montanide™ adjuvants on protective host immunity. In the absence of profilin immunization, *E. acervulina* infection decreased body weight gain between 0 and 10 d post-infection by approximately 100 g/bird compared with uninfected animals ($p < 0.05$; Fig. 2A). Vaccination with 50 µg of profilin alone did not restore weight gain to the

Table 1

Oligonucleotide primers used for quantitative RT-PCR of chicken cytokine transcripts.

RNA target	Primer sequences	PCR product size (bp)	Accession no.
GAPDH			
Forward	5'-GGTGGTGCTAAGCGTGTTAT-3'	264	K01458
Reverse	5'-ACCTCTGCATCTCTCCACA-3'		
IL-2			
Forward	5'-TCTGGGACCACTGTATGCTCT-3'	256	AF000631
Reverse	5'-ACACCAGTGGGAAACAGTATCA-3'		
IL-10			
Forward	5'-CGGGAGCTGAGGGTGAA-3'	272	AJ621614
Reverse	5'-GTGAAGAAGCGGTGACAGC-3'		
IFN- γ			
Forward	5'-AGCTGACGGTGGACCTATTATT-3'	259	Y07922
Reverse	5'-GGCTTTGCGCTGGATTTC-3'		
IL-17A			
Forward	5'-CTCCGATCCCTTATTCTCCTC-3'	292	AJ493595
Reverse	5'-AAGCGGTTGTGGTCTCAT-3'		
TGF- β 4			
Forward	5'-CGGGACGGATGAGAAGAAC-3'	258	M31160
Reverse	5'-CGGCCACGTAGTAATGAT-3'		
IL-15			
Forward	5'-TCTGTTCTTCTGTTCTGAGTGATG-3'	243	AF139097
Reverse	5'-AGTGATTGCTTCTGCTTTGGTA-3'		

level seen in uninfected chickens, as reported earlier (Ding et al., 2004). However, weight gains in animals immunized with 50 μ g of profilin plus ISA 70 VG or ISA 71 VG were significantly greater ($p < 0.05$) than those of infected chickens that were non-vaccinated or vaccinated with profilin alone. Indeed, body weight gains in the profilin plus ISA 70 VG or ISA 71 VG groups were restored to the level that was seen in the uninfected control group.

No fecal oocyst shedding was observed in uninfected controls (data not shown). Oocyst shedding in *E. acervulina*-infected, non-vaccinated chickens was approximately 3.0×10^8 oocysts/bird (Fig. 2B). Birds immunized with profilin alone exhibited significantly reduced oocyst shedding ($\sim 2.0 \times 10^8$ oocysts/bird) that was further reduced ($\sim 1.0 \times 10^8$ oocysts/bird; $p < 0.05$) in animals given profilin plus ISA 71 VG (Fig. 2B).

3.2. Effects of vaccination with profilin plus MontanideTM adjuvants on profilin serum antibody responses

Prior studies showed that the profilin subunit vaccine induces a robust humoral immune response against the immunogen (Ding et al., 2004). Therefore, profilin serum antibody levels following primary immunization were measured in chickens given profilin alone or profilin plus adjuvants. Following primary immunization, profilin-specific antibody titers were approximately 2-fold greater ($p < 0.05$) when the antigen was administered with any of five of the adjuvants tested compared with animals immunized with profilin alone (Fig. 3A). Similarly, following secondary immunization, all adjuvants with the exception of ISA 206 VG increased profilin antibody levels compared with immunization in the absence of adjuvant ($p < 0.05$; Fig. 3B).

3.3. Effects of vaccination with profilin plus MontanideTM adjuvants on cytokine transcript levels in intestinal IELs

Immunization with the profilin subunit vaccine has previously been shown to increase the levels of mRNAs encoding proinflammatory as well as anti-inflammatory cytokines in intestinal IELs (Ding et al., 2004; Lillehoj et al., 2005a). Therefore, we next evaluated the adjuvant effects on profilin-stimulated cytokine gene expression. Vaccination with profilin plus ISA 201 VG increased the levels of transcripts encoding the cytokine IL-2, while vaccination with profilin plus ISA 70 VG increased transcripts for proinflammatory IL-17A, in intestinal IELs compared with vaccination with profilin alone ($p < 0.05$; Fig. 4). Interestingly, profilin plus ISA 70 VG, ISA 71 VG, or ISA 206 VG also increased mRNAs for the anti-inflammatory IL-10. By contrast, profilin plus ISA 70 VG and ISA 71 VG, decreased the levels of IL-15 transcripts. The broadest effect of the adjuvants was seen on IFN- γ transcripts, which were increased by immunization with profilin plus ISA 70 VG, ISA 71 VG or ISA 201 VG compared with profilin alone.

3.4. Effects of vaccination with profilin plus MontanideTM ISA 71 VG adjuvant on recruitment of CD8⁺ T cells to the site immunization

Previous studies have demonstrated that CD8⁺ cytotoxic T lymphocytes are recruited to the skin following subcutaneous immunization with intradermal peptide subunit vaccines (Chen et al., 2005). Effect of ISA 71VG on lymphocyte trafficking to the site of vaccination was evaluated using mouse monoclonal antibodies detecting various macrophages and lymphocyte subpopulations using confocal microscopy. As shown in Fig. 5, in response to profilin plus ISA 71VG vaccination, high number of infiltrating

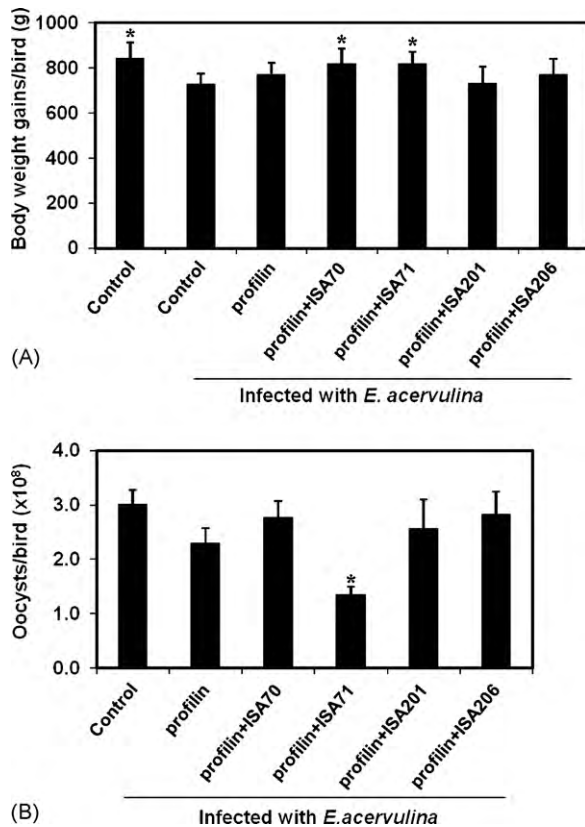


Fig. 2. Effect of recombinant profilin antigen vaccination in combination with Montanide™ adjuvants on resistance to experimental coccidiosis. Chickens were immunized subcutaneously with PBS (control) or 50 μ g recombinant profilin protein alone or emulsified in the indicated adjuvants and non-infected or infected with 1.0×10^4 *Eimeria acervulina* oocysts and body weight gains (A) and fecal oocyst shedding (B) were determined. Each bar represents the mean \pm S.D. value ($N=5$). * $p < 0.05$ comparing vaccinated group with non-vaccinated (PBS) group according to the Tukey's test.

lymphocytes K55 (Fig. 5B), particularly CD8⁺ lymphocytes (Fig. 5C), was observed whereas few K1⁺, TCR $\gamma\delta$ ⁺ and TCR $\alpha\beta$ ⁺ cells (data not shown) were seen in the dermis around the perivascular areas and the areas close to the epidermis.

4. Discussion

The current study used a low-dose *Eimeria* profilin subunit vaccine to evaluate the efficacy of Montanide™ series adjuvants to afford protection against a high-dose, oral challenge infection with live sporulated *E. acervulina* oocysts. The results demonstrated that: (1) immunization with profilin plus ISA 70 VG or ISA 71 VG increased body weight gains in *E. acervulina*-infected chickens compared with vaccination with profilin alone, (2) profilin plus ISA 71 VG reduced fecal oocyst shedding compared with adjuvant-free vaccination, (3) all adjuvants enhanced profilin serum antibody levels, (4) increased levels of gene transcripts encoding IL-2, IL-10, IL-17A, and IFN- γ , but decreased levels of IL-15 transcripts, were seen in intestinal IELs of chickens immunized with profilin plus adjuvants

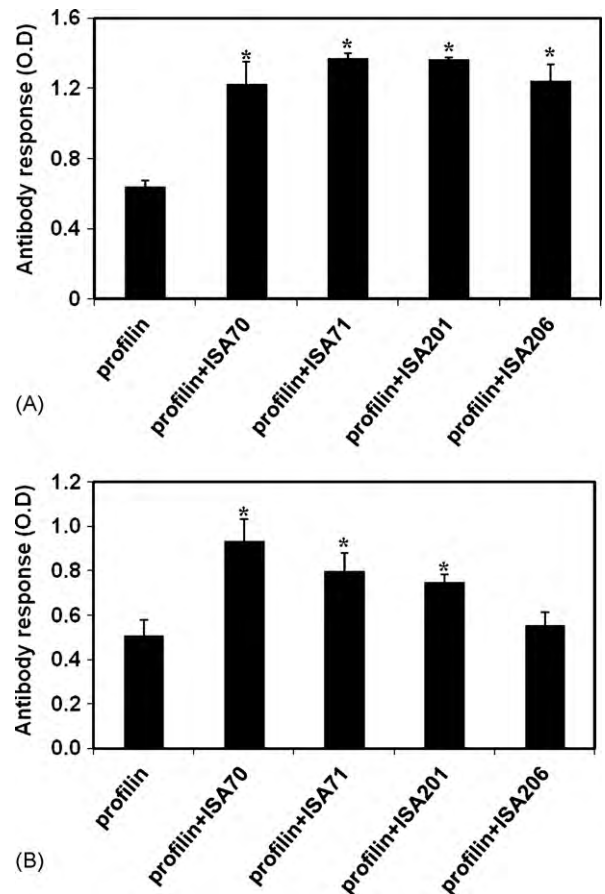


Fig. 3. Effect of recombinant profilin antigen vaccination in combination with Montanide™ adjuvants on profilin serum antibody levels. Chickens were immunized subcutaneously with 50 μ g recombinant profilin protein alone or emulsified in the indicated adjuvants at 7 and 14 d and profilin antibody levels were determined by ELISA at 7 d post-primary (A) and 7 d post-secondary (B) immunization. Each bar represents the mean \pm S.D. value ($N=3$). The asterisk (*) indicates significantly increased antibody levels comparing profilin plus adjuvant group with profilin alone group.

compared with profilin alone, and (5) increased infiltration of CD8⁺ lymphocytes at the site of subcutaneous immunization was observed in chickens given profilin plus ISA 71 VG compared with profilin alone.

Due to the increasing regulations and bans on the use of anticoccidial drugs in farm animals, a lot of alternative control strategies including recombinant protein, subunit, live-vector or DNA vaccination strategies have been tried for the control of avian coccidiosis (Ding et al., 2008; Shirley et al., 2007). Accumulating evidence indicates that weak immunogenicity of currently used subunit vaccines against many infectious diseases of livestock and poultry increases production cost and fails to effectively curtail disease outbreaks. Particularly in the poultry industry, repeated immunizations are often necessary to generate a high level of immunity to the most common pathogens commonly encountered in commercial flocks. The limited availability of well-characterized vaccine adjuvants further hinders the struggle against many economically important avian diseases. The recent availability of new adjuvant formulations,

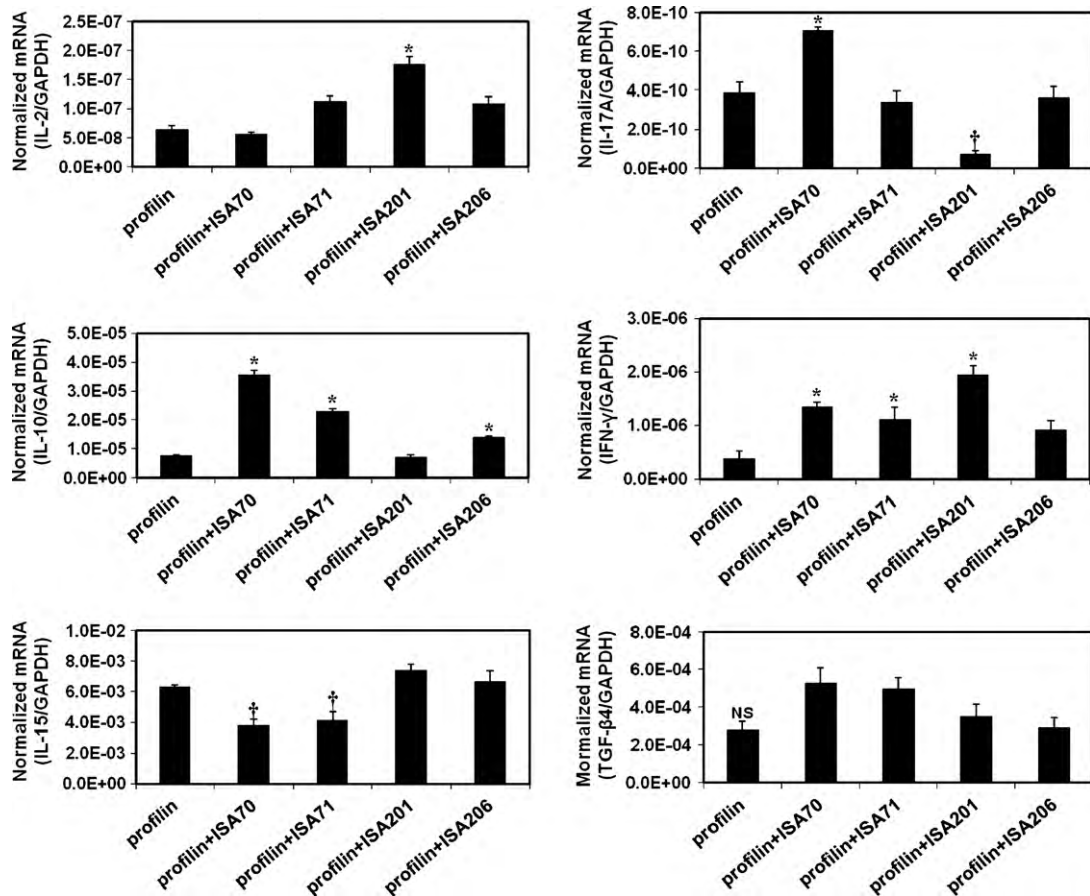


Fig. 4. Effect of recombinant profilin antigen vaccination in combination with Montanide™ adjuvants on intestinal IEL cytokine mRNA levels. Chickens were immunized subcutaneously with 50 µg recombinant profilin protein alone or emulsified in the indicated adjuvants at 7 and 14 d and IEL cytokine levels were determined by quantitative RT-PCR at 7 d post-secondary immunization. Each bar represents the mean \pm S.D. value ($N = 3$). The asterisk (*) indicates significantly increased mRNA levels comparing profilin plus adjuvant group with profilin alone group. The dagger (†) indicates significantly decreased mRNA level comparing profilin plus adjuvant group with profilin alone group. NS indicates not significant difference between comparing profilin plus adjuvant group with profilin alone group.

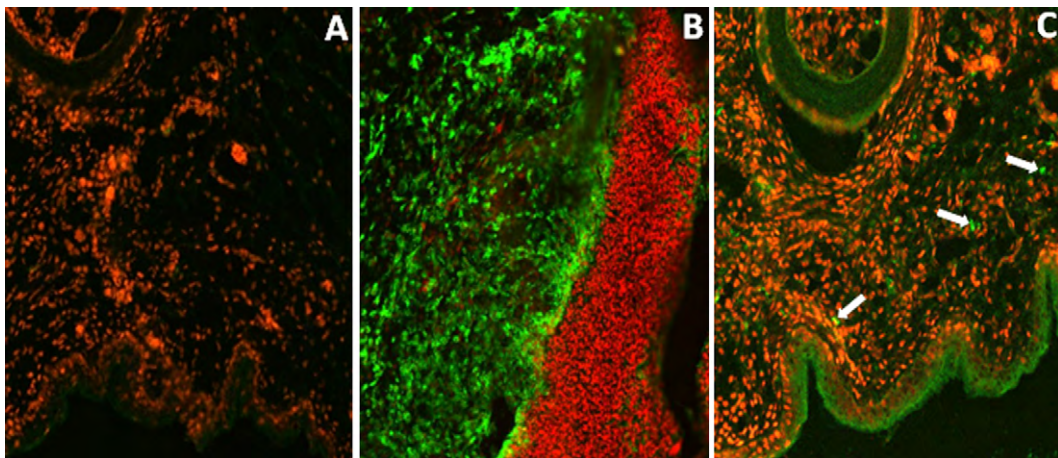


Fig. 5. Effect of recombinant profilin antigen vaccination in combination with Montanide™ adjuvants on K55 or CD8⁺ lymphocyte staining at the site of injection. Chickens were immunized subcutaneously with 50 µg recombinant profilin protein alone (A) or emulsified in ISA 71 VG (B and C) at 7 and 14 d. K55 cells (B) or CD8⁺ cells (C; arrows) were visualized in the skin of chickens vaccinated with profilin plus Montanide™ adjuvants by indirect immunofluorescence. No CD8⁺ cells were observed in the skin of profilin-vaccinated chickens (A).

such as second-generation water-in-oil (W/O), water-in-oil-in-water (W/O/W), or oil-in-water (O/W) emulsions with proven efficacy for subunit vaccines in animal models, promises to add to the armamentarium used against infectious diseases in poultry production (Gupta et al., 1995; Belloc et al., 2008).

Currently, the most commonly used adjuvants for veterinary vaccines are aluminum hydroxide as well as W/O, W/O/W, and O/W emulsions (Gupta et al., 1995; Bowersock and Martin, 1999). The Montanide™ ISA series of adjuvants are blends of oil and surfactants. These types of vaccines have been used in veterinary applications for over 25 years and safely tested in human clinical trials (Aucouturier et al., 2006). According to the adjuvant composition and formulation process, the vaccine formulated can be W/O, O/W or W/O/W emulsions. Montanide™ ISA 201 VG and ISA 71 VG are mineral oil-based adjuvants that have been, respectively developed for the manufacture of W/O/W and W/O emulsions. Both comprises a specific enriched light mineral oil ensuring an improvement of the cellular response and an extremely refined emulsifier obtained from mannitol and purified oleic acid of vegetable origin. Comparatively, Montanide™ ISA 206 VG and ISA 70 VG also allow the manufacture of W/O/W and W/O but comprise a high grade injectable mineral oil developed not to trigger specifically a cellular set of response (Cox et al., 2003; Aucouturier et al., 2006). These adjuvants can be selected to avoid the commonly seen side effects that are associated with other mineral oil emulsion, such as incomplete Freund's adjuvant which may produce inflammatory reactions, granulomas, and ulcers at the injection site (Gupta et al., 1993; Leenaars et al., 1998; Aucouturier et al., 2006). Indeed, Montanide™ adjuvants have been used with experimental vaccines in mice, rats, guinea pigs, chickens, and sheep using natural, recombinant, and synthetic antigens (Iyer et al., 2001; Cox et al., 2003; Harrington et al., 2009). In humans, Montanide™ adjuvants have been used in trial vaccines against HIV, malaria, and breast cancer (Petrovsky and Aguilar, 2004).

It is generally accepted that body weight gain and fecal oocyst shedding are reliable clinical signs for the evaluation of vaccine efficacy and protective immunity in avian coccidiosis (Lee et al., 2007). Both parameters are positively correlated with the appearance of intestinal immune IELs as well as gut proinflammatory cytokines and chemokines in chickens immunized with a variety of different *Eimeria* vaccines (Lillehoj and Lillehoj, 2000; Hong et al., 2006). Of note in this investigation, IFN- γ gene transcripts were significantly increased in chickens vaccinated with profilin plus all of the adjuvants tested, with the exception of ISA 206 VG, compared with immunization with profilin alone. IFN- γ is a major cytokine which is induced early during infection with *Eimeria* parasites and that exerts a direct inhibitory effect of on intracellular parasite development (Lillehoj and Choi, 1998; Lillehoj and Lillehoj, 2000; Hong et al., 2006). Immunization with profilin plus selected ISA adjuvants also stimulated the expression of the IL-2 and IL-17A cytokines. These cytokines are belonging to the cellular set of the immune response (Th1). These finding indicates that the cellular response was activated, indicating a stronger Th1 profile

of response when profiling was associated to ISA 71 VG. Because Ding et al. (2004) reported that recombinant IL-2 and IL-17A, along with other chicken cytokines, had an adjuvant-like effect themselves on enhancing protective immunity to avian coccidiosis when coadministered with the profilin vaccine, it is possible that an amplification loop involving augmented proinflammatory cytokine production may be activated with the use of the Montanide™ adjuvants.

It is important to note, however, that the ISA range adjuvants also increased the expression of the anti-inflammatory IL-10 and decreased expression of the IL-15. While it may appear counterintuitive that both pro- and anti-inflammatory cytokines are simultaneously up-regulated by vaccination in the presence of adjuvant, it is currently unclear what role the individual immune mediators play in the overall protective immune response during coccidiosis. Future studies are needed to address the kinetics of expression of the individual cytokines, their sites of production in the intestine, and relative bioactivities during experimental *Eimeria* infection *vis-à-vis* the natural conditions of field infection.

One of the primary functions of cytokines and chemokines is to recruit and amplify immune effector cells to sites of infection. In the case of avian coccidiosis, an important role for cytokine-producing intestinal CD8⁺ T lymphocytes and NK cells in mediating protective immunity is well-established (Lillehoj and Trout, 1994). In murine experimental models of apicomplexan parasite infections, the critical roles of CD4⁺ and CD8⁺ T cells, both as effector cells and local producers of cytokines that regulate host responses, have been documented (Sher and Coffman, 1992). In the current investigation, although no adverse local inflammatory reactions were observed macroscopically at the site of subcutaneous adjuvant administration, a large increase in the infiltration of CD8⁺ lymphocytes was observed. Given the vital function of CD8⁺ and other immune cells, such as dendritic cells, in the skin of mammalian and avian species (Mackay, 1993; Caux et al., 1995; Del Cacho et al., 2009), this observation may be especially relevant to the increased induction of protective immunity induced by the Montanide™ adjuvants.

In summary, this report is the first to document the efficacy of Montanide™ adjuvants in conjunction with the *Eimeria* recombinant profilin antigen for vaccination against avian coccidiosis. The results of this study suggest that vaccination with the *E. acervulina* profilin subunit vaccine in combination with Montanide™ adjuvants augments protective immunity against avian coccidiosis. Future studies on the effects of these adjuvants on cytokine production and the role of various infiltrating cells on enhanced protective immune response against coccidiosis will shed important insights on their mechanisms of action and applicability to other poultry diseases.

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